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TO ALL WHOM IT MAY CONCERN:

Be it known that WE, Heidi Stuhlmann, Jing-Wei Xiong, and Mark B. Taubman, citizens of Germany, People's Republic of China, and the US, respectively, residing in New York County,New York County, and Westchester County, respectively, all in the State of New York, having post office addresses of 200 East 94th Street, Apt. 1514, New York, New York 10128; 306 East 96th Street, Apt. 3G, New York, New York, 10128; and 5 Jochum Avenue, Larchmont, New York 10538, respectively, have invented an improvement in

VASCULAR ENDOTHELIAL ZINC FINGER 1 GENE AND PROTEIN AND USES THEREOF

of which the following is a

SPECIFICATION

The present invention involves subject matter developed under National Institute of Health Grant No. 1R29 HD 31534, so that the United States Government may have certain rights herein.

1. INTRODUCTION

The present invention relates to the diagnosis and treatment of circulatory system and vascular disorders, and to assays for the identification of agents which act upon the circulatory system. It is based, at least in part, on the identification of a novel mouse gene, termed *Vezf1* (for "Vascular endothelial zinc finger 1"), which is expressed at higher levels during embryonic development of the circulatory system, in damaged blood vessels, and in newly formed blood vessels associated with tumor growth.

2. BACKGROUND OF THE INVENTION

2.1. FORMATION OF BLOOD VESSELS DURING EMBRYONIC DEVELOPMENT

The embryonic vascular system develops by two distinct processes, termed vasculogenesis and angiogenesis (Breier and Risau, 1996, Trends Cell Biol.6:454-456; Folkman and D'Amore, 1996, Cell 87:1153-1155; Risau, 1997, Nature 386:671-674). Both are complex multistep processes involving remodeling of extracellular matrix and regulating the proliferation, migration, differentiation, and assembly of endothelial cells to produce, modify, and maintain the complex networks of arteries and veins which constitute the circulatory system.

"Vasculogenesis" is the term applied to the process whereby endothelial cells differentiate *de novo* from mesodermally derived precursors in the embryo and assemble into primitive blood vessels. These precursor cells, called "angioblasts", are vascular endothelial cells which have not yet organized to form a lumen. Genetic and developmental studies suggest the existence of a bipotential precursor cell, the "hemangioblast", that gives rise to both angioblasts and hematopoietic cells (Kennedy et al., 1997, Nature 386:488-492; Shalaby et al., 1995, Nature 376: 62-66). In vertebrates, angioblasts first arise at the early headfold stage in the extraembryonic yolk sac. These angioblasts form by aggregation of splanchnic mesodermal cells into angiogenic clusters, or "blood islands". They, in turn, differentiate into double-walled tubes having an inner wall formed by flat endothelial cells, which create a cellular lining for the resulting blood vessel, and an outer wall formed by smooth muscle cells. The central cells of the blood islands differentiate into embryonic hematopoietic cells. Differentiation and growth of the blood islands eventually give rise to a capillary network that drains into the two vitelline veins of the embryo. In addition, vasculogenesis also occurs in the embryo itself (as opposed to the yolk

sac). Angioblasts arise in the paraxial and lateral mesoderm of the embryo and give rise to primordia of the heart, the dorsal aorta, and large vessels of the head, lung, and gastrointestinal system.

"Angiogenesis" is a process involving the maturation and remodelling of the primitive vascular plexus into a complex network of large and small vessels. In addition, angiogenesis leads to sprouting of pre-existing vessels into intersomitic spaces and initially avascular organs, such as kidney, brain, and limb buds (Coffin and Poole, 1991, Anat. Rec. 231:383-395).

2.2. ADULT ANGIOGENESIS

After the circulatory system has been fully formed, angiogenesis is required for the normal growth of postnatal tissues, and continues throughout adult life, accounting for transient phases of neo-vascularization during the normal female menstrual cycle, during pregnancy in the placenta, and during wound healing (Folkman, 1995, Nature Med. 1: 27-31; Folkman and Shing, 1992, J. Biol. Chem. 267:10931-10934; Risau, 1997, Nature 386:671-674). As in embryonic development, angiogenesis in the adult involves sprouting and growth of pre-existing blood vessels, as well as remodeling (Risau, 1997, Nature 386:671-674).

Postnatally, angiogenesis has also been implicated in a number of diseases and disorders, in which abnormal endothelial growth may be either the primary cause of, or indirectly caused by, the pathological process. For example, abnormal endothelial cell proliferation has been postulated as playing a role in atherosclerosis. Further, the development of blood vessels in neoplasms plays a role in tumor growth and metastasis, and deregulated angiogenesis has been

implicated in pathological processes such as rheumatoid arthritis, retinopathies, hemangiomas, and psoriasis (Folkman, 1995, Nature Med. 1: 27-31; Hanahan and Folkman, 1996, Cell 86:353-364).

2.3 MOLECULAR MECHANISMS OF VASCULOGENESIS AND ANGIOGENESIS

The genetic and molecular mechanisms that control development of the vascular system are beginning to be understood (reviewed in Folkman and D'Amore, 1996, Cell 87:1153-1155; Risau, 1997, Nature 386:671-674). Generation of angioblasts from paraxial and lateral plate mesoderm requires the function of members of the fibroblast growth factor family. Recent evidence suggests that vascular endothelial growth factor ("VEGF") and its cellular receptors (*VEGFR-1* or *flt-1* and *VEGFR-2* or *flk-1*) are essential for vasculogenesis during embryogenesis. VEGF, which is related to platelet-derived growth factor ("PDGF"), is secreted by the endoderm and functions in a paracrine manner (Breier et al., 1992, Development 114: 521-532; Breier and Risau, 1996, Trends Cell Biol. 6:454-456), whereas its tyrosine kinase receptors are co-expressed by the mesoderm-derived angioblasts and endothelial cells (Fong et al., 1995, Nature 376:66-70; Millauer et al., 1993, Cell 72:835-846; Yamaguchi et al., 1993, Development 118:489-498). Disruption of the gene for *VEGFR-2* (*flk-1*) in mice results in a block to differentiation of angioblasts into endothelial cells and leads to embryonic death at day 8.5-9.5 (Shalaby et al., 1995, Nature 376:62-66). Disruption of the gene for the other receptor, *VEGFR-1* (*flt-1*), leads to abnormal assembly of endothelial cells in blood vessels and embryonic death at day 9 of development (Fong et al., 1995, Nature 376: 66-70). Inactivation of a single VEGF allele leads to abnormal vascular structures and is lethal in the mouse embryo at about day 11 (Carmeliet et al.,

1996, Nature 380:435-439; Ferrara et al., 1996, Nature 380:439-442). Therefore, each of these components of the VEGF signaling pathway is essential for vascular development and there is no functional redundancy among the ligand or its receptors. VEGF signal transduction involves receptor tyrosine kinase autophosphorylation, activation of phosphoinositol 3-kinase, phospholipase C γ and MAP kinase (Breier and Risau, 1996, Trends Cell Biol. 6:454-456). However, endothelial cell specific genes that lie downstream in the VEGF/*flk-1*/*flt-1* signaling pathway have not yet been conclusively identified.

Normal angiogenesis is thought to depend on a balance between inducers and inhibitors of endothelial cell proliferation and migration (Hanahan and Folkman, 1996, Cell 86:353-364). Positive regulatory molecules include VEGF and angiopoietin-1 ("*Ang1*"; Davis et al., 1996, Cell 87: 1161-1169). *Ang-1* signals through the tyrosine kinase receptor *Tie2/Tek* that is specifically expressed on endothelial cells and early hematopoietic cells (Dumont et al., 1992, Oncogene 7:1471-1480). Absence of *Ang1*, its receptor *Tie2/Tek*, or *Tie1*, a closely related orphan receptor, causes severe abnormalities of the vascular system in both mouse and human. These findings suggest that these genes and their products play a role in angiogenesis and remodeling that occurs subsequent to VEGF and its receptors (Sato et al., 1995, Nature 376:70-74; Suri et al., 1996, Cell 87:1171-1180; Vikkula et al., 1996, Cell 87:1181-1190). Recently, Angiopoietin-2 ("*Ang2*"), a factor closely related to *Ang1*, has been identified and shown to function as a natural antagonist for *Ang1* and its *Tie2* receptor (Maisonpierre et al., 1997, Science 277:55-60). Other inhibitors of angiogenesis include angiostatin, an internal fragment of plasminogen (O'Reilly et al., 1994, Cell 79:315-328) and endostatin, a C-terminal fragment of collagen XVIII (O'Reilly et al., 1997, Cell 88:277-285), both of which were isolated from

tumors. However, their precise roles in normal angiogenesis and their relationship to the *Angl/Tie2* signaling pathway remain unknown.

2.4 MODEL SYSTEMS FOR STUDYING ENDOTHELIAL CELL DIFFERENTIATION

Several different model systems have been developed to study vasculogenesis and angiogenesis. The most common *in vivo* models are the chick embryo chorioallantoic membrane ("CAM") and the rabbit retina. More recently, *in vitro* cultures of embryonic stem ("ES") cell derived embryoid bodies have been used to study differentiation of angioblasts and the formation of vascular channels (Wang et al., 1992, Development 14:303-316). Finally, hemangioblastoma or yolk sac-derived endothelial cell lines have been established that form capillary tubes when grown in matrix-containing gels (Obeso et al., 1990, Lab. Invest. 63:259-269; Wang et al., 1996, Devel. Biol. Anim. 32:292-299).

2.5 THE HUMAN GENE DB1

The present invention relates to the discovery of a novel mouse gene, *Vezf1*, expressed in association with angiogenic and vasculogenic events. Sequencing of the cDNA of *Vezf1* revealed that it encoded a protein with over 98 percent homology to the previously identified human gene DB1 (Koyano-Nakagawa et al., 1994, Mol. Cell. Biol. 14:5099-5107). DB1 protein, a 516 amino acid protein with six zinc finger motifs, was found to be widely expressed in human blood cells and adult organs (*Id.*). It was identified in the Jurkat T cell line as a nuclear factor with sequence-specific DNA binding to a CT/GC-rich region of the promoter of the interleukin 3 ("IL-3") gene (*Id.*). Any role for DB1 in endothelial cell development,

angiogenesis, or vasculogenesis had not been known prior to the present invention. Rather, since no transcriptional activation of the IL-3 promoter could be detected, the *in vivo* function of DB1 remained elusive.

3. SUMMARY OF THE INVENTION

The present invention relates, at least in part, to the discovery of a novel mouse gene, referred to herein as *Vezf1* (for "Vascular endothelial zinc finger 1"), which is expressed in association with vasculogenesis and angiogenesis.

In a first series of embodiments, the present invention provides for a nucleic acid encoding *Vezf1*, and a *Vezf1* protein having an amino acid sequence as encoded by that nucleic acid.

In a second set of embodiments, the present invention provides for a method of identifying an endothelial cell, comprising identifying the expression of a *Vezf1*-encoding RNA or a *Vezf1* protein in the cell.

In a third series of embodiments, the present invention provides for a method of diagnosing a vascular disorder in a subject, whereby the presence, amount, and/or molecular characteristics of the *Vezf1* gene, its corresponding mRNA or a cDNA thereof, or its product protein are determined and compared to normal values. Differences in the molecular characteristics of the *Vezf1* gene or its expression would support a diagnosis of a vascular disorder. In a related set of embodiments, subjects carrying a defective *Vezf1* gene may be identified according to the invention; such individuals may be at risk of developing a vascular disorder or passing such a condition to their progeny.

In a fourth set of embodiments, the present invention provides for a non-human animal model system for studying angiogenesis and vasculogenesis, and in particular, the role of *Vezf1* in these processes. Such models may be used to study the effects of systemic or local increases in *Vezf1* expression, or, alternatively, decreased or aberrant *Vezf1* expression. Such model systems, or cell lines derived therefrom, may be used to identify agents that act as agonists or antagonists of *Vezf1* activity.

In a fifth set of embodiments, the present invention provides for methods of treating vascular diseases and disorders. For example, but not by way of limitation, the inhibition of *Vezf1* expression may be used to inhibit angiogenesis, for example, in a neoplasm. Alternatively, increased expression of *Vezf1* may be useful in promoting wound healing or in treating clinical disorders related to ischemia.

4. DESCRIPTION OF THE FIGURES

FIGURE 1. Nucleic acid sequence of mouse *Vezf1* cDNA (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2).

FIGURE 2. Nucleic acid sequence of human DB1 cDNA (SEQ ID NO:3) and deduced amino acid sequence (SEQ ID NO:4) (Koyano-Nakagawa et al., 1994, Mol. Cell. Biol. 14:5099-5107; GeneBank Acc. No. D28118).

FIGURE 3. Schematic overview of a genetic screen for developmentally regulated genes using retroviral entrapment vectors with human alkaline phosphatase ("AP") as a reporter gene.

FIGURE 4A-X. Differential AP reporter gene expression during *in vitro*

differentiation of ES cell clones with entrapment vector insertions. ES cells from individual clones were induced to spontaneously differentiate into embryoid bodies. Aliquots from day-4, day-7, day-10 embryoid bodies, or terminally differentiated cultures, respectively, were stained for AP activity with BCIP + NBT (A-L) or BM purple (Boehringer Mannheim) (M-X), and photographed using a Nikon SMZ-U stereomicroscope, as follows:

(A,B): day-4,day-7 embryoid bodies originating from uninfected ES cells;

(C,D): day-4, day-10 embryoid bodies originating from PT-AP clone IV E3-8;

(E,F): day-4, day-10 embryoid bodies originating from PT-AP clone 7-65;

(G,H): day-4, day-7 embryoid bodies originating from PT-IRES-AP clone 1-13;

(I,J): day-7, day-10 embryoid bodies originating from PT-IRES-AP clone 1-58;

(K,L): day-7, day-10 embryoid bodies originating from PT-IRES-AP clone 2-86;

(M,N): day-7, day-10 embryoid bodies originating from PT-IRES-AP clone 2-51;

(O,P): day-7, day-10 embryoid bodies originating from PT-IRES-AP clone 4-45;

(Q,R): day-7, day-10 embryoid bodies originating from PT-IRES-AP clone 6-33;

(S,T): day-4, day-10 embryoid bodies originating from PT-IRES-AP clone 7-26;

(U,V): day-7, re-plated embryoid bodies originating from PT-IRES-AP clone 9-E5; and

(W,X): day-7, day-10 embryoid bodies originating from PT-IRES-AP clone 9-G7.

FIGURE 5 A-D. AP expression in ES cells of the #1-13 clone. (A) Day-4 embryoid bodies in suspension culture. (B) Day-7 embryoid bodies in suspension culture. (C) Day-10 embryoid bodies in suspension culture. (D) Re-plated day-4 embryoid body monolayers in gelatin-coated culture dishes.

FIGURE 6A-B. (A) Retroviral vector insertion #1-13 with flanking genomic

DNA. (B) Southern analysis, using a 0.7 kb genomic DNA fragment 3' of the insertion's EcoRI site as a probe, of high molecular weight DNA from R1 ES cells, cells of the #1-13 insertion-bearing ES cell clone, and transgenic mice homozygous (-/-) or heterozygous (+/-) for the #1-13 insertion. DNA from wild-type CD-1 (+/+) mice lacking the #1-13 mutation was used as a control, and λ DNA digested with EcoRI and BamHI was used as a size marker (left lane).

FIGURE 7. Amino acid sequence alignment of residues 168-360 of mouse *Vezf1* (SEQ ID NO:12) and residues 275-469 of mouse Pur-1 (SEQ ID NO:13).

FIGURE 8. Retroviral insertion in the 3' untranslated region of *Vezf1* in ES clone #1-13. Probes A, B and C correspond to cDNA clones of *Vezf1*, probe A corresponding to nucleotides 1-776, probe B corresponding to nucleotides 97-1444, and probe C corresponding to nucleotides 1397-2820.

FIGURE 9(A-F). Expression of AP in developing embryos containing the #1-13 retroviral insertion, as analyzed by histochemical staining with BM purple (Boehringer Mannheim). Developmental stages studied were (A) E7.25, (B) E7.5, (C) E8.5, (D) E9.5, (E) E10.5 and (F) E11.5.

FIGURE 10(A-H). Expression of AP in histological sections of developing embryos containing the #1-13 retroviral insertion, as measured by histochemical staining. Developmental stages studied were (A) E7.25 (14X magnification), (B) E7.25 (100X magnification), (C) E7.25 (50X magnification), (D) E7.25 (200X magnification), (E) E7.5 (50X magnification), (F) E7.5 (100X magnification), (G) E8.0 (50X magnification) and (H) E8.0 (200X magnification).

FIGURE 11A-H. Expression of AP in histological sections of embryos containing

the #1-13 retroviral insertion at stages E8.5 (A-D) and E9.5 (E-H) measured by histochemical staining, showing expression in (A and E) the whole embryo, (B) the head fold, (C) the heart, (D and H) the yolk sac, (F) the neural tube; and (G) the cardiac ventricle.

FIGURE 12 (A-J) Expression of *Vezf1* mRNA in developing embryos as measured by *in situ* hybridization. Developmental stages and tissues evaluated were (A) E7.25 (dark field), (B) E7.25 (bright field), (C) E8.5 (dark field), (D) E8.5 (bright field), (E) E9.5 (dark field), (F) dorsal aorta (dark field), (G) somites (dark field), (H) heart (dark field), (I) lung (bright field) and (J) kidney (bright field).

FIGURE 13A-C. Northern blot analysis of *Vezf1* RNA expression in (A) various stages of embryoid bodies, ES cells, and mouse embryos; (B) in hematopoietic and endothelial cell lines; and (C) in various tissues of adult mice.

FIGURE 14A-B. Southern analysis of hybridization of *Vezf1* probe with DNAs from various species restricted with either BamHI (lanes marked "B"), EcoRI (lanes marked "R"), or HindIII (lanes marked "H").

FIGURE 15A-B. Map of Jackson BSS chromosome 2 showing location of the *Vezf1* gene. A DNA panel from the Jackson Laboratory community resource (C57BL/6J x *M. spretus*), BSS interspecific backcross, was used to map the *Vezf1* locus using PCR techniques. (A) Haplotype figure from the Jackson backcross showing part of Chromosome 2 with loci linked to *Vezf1* (black boxes: C57BL6/JEi allele). (B) Map figure from the Jackson BSS backcross showing the proximal end of Chromosome 2. The map is depicted with the centromere toward the top. Raw data from the Jackson Laboratory were obtained from the World Wide Web address http://www.jax.org/resources/documents/cmdata.

FIGURE 16. Restriction enzyme map of 20 kb mouse genomic fragment containing the promoter element and first two exons.

FIGURE 17. Structure of plasmid vectors expressing wild type and mutant *Vezf1*. The open box (top) depicts the *Vezf1* cDNA from nucleotides 1 to 2280 (ATG transcriptional start at nucleotide 39) with coding regions for six putative zinc fingers (indicated by black bars). DZF-*Vezf1* contains a deletion from nucleotide 309 to 992, including zinc fingers 2 to 6 and half of the first zinc finger. *Vezf1*-ZF contains nucleotides 309 to 992 of the cDNA including zinc fingers 2 to 6 and half of the first zinc finger.

FIGURE 18. Retroviral entrapment vectors. The depicted constructs contain a 1.9 kb fragment of the human placental alkaline phosphatase gene (AP) as a reporter gene.GT-AP contains the Mo-MLV *env* splice acceptor site (SA) and PT-IRES-AP contains a 0.6 kb IRES fragment from encephalomyocarditis virus (EMCV). All constructs contain a 1.3 kb *neo* expression cassette with a PGK promoter and the neo^r gene. The transcriptional orientation of the AP gene and the *neo* expression cassette are indicated by arrows. The vectors contain 5' Mo-MLV sequences up to nucleotide 1560 derived from pMov9, including the 5'LTR, the packaging site Ψ, and 1 kb of gag sequences. The 3' end of the vectors contain Mo-MLV sequences from nucleotide 7674, including the 3' LTR with the 200 bp *E. coli sup F* gene inserted at position 7911 in U3, derived from $pLTR^{sup-1}$. A 294 bp deletion was generated in $pLTR^{sup-1}$ from PvuII (position 7935) to SacI (position 8229) containing the retroviral enhancer and promoter sequences. The relative position of the *supF* insert, the deletion of enhancer and promoter (enh^-/pro^-) and the polyadenylation [p(A)] sequence are indicated. The length of different fragments and the relevant restriction sites used for the construction of the vectors are shown. E=EcoRI;

K=KpnI; X=XhoI.

5. DETAILED DESCRIPTION OF THE INVENTION

For purposes of clarity, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

(i) the *Vezf1* gene and its products;

(ii) diagnostic methods;

(iii) model systems; and

(iv) methods of treatment.

5.1 THE *VEZF1* GENE AND ITS PRODUCTS

The present invention relates to a *Vezf1* gene (including its control elements), RNA transcribed from the *Vezf1* gene or any cDNA or antisense counterparts thereof, and its encoded protein. The term "*Vezf1* gene", as used herein, collectively includes the mouse *Vezf1* gene, its human counterpart (also referred to as DB1), and homologs thereof in other species which are at least about 90 percent, and preferably about 95 percent, homologous to the mouse *Vezf1* gene (homology determined by MACVECTOR, Blast Search Algorithm (Netscape Navigator, 3.01), and/or which hybridize to the mouse *Vezf1* gene under stringent hybridization and wash conditions, such as hybridization in 50 percent formamide, 5x SSPE, 2x Denhardt's, 0.5 percent SDS, 100 μg/ml salmon sperm DNA, at 37°C; and washes in 0.2 x SSC, 0.1 percent SDS at 50°C.The term "*Vezf1* cDNA", as used herein, collectively includes the mouse *Vezf1* cDNA having a sequence as set forth in FIGURE 1 (SEQ ID NO:1), its human counterpart

(referred to herein as DB1), having a nucleic acid sequence as set forth in FIGURE 2 (SEQ ID NO:3) and homologs thereof in other species which are at least about 90 percent, and preferably about 95 percent, homologous to mouse *Vezf1* cDNA (as determined by MACVECTOR, see above) and/or which hybridize to the mouse *Vezf1* gene under stringent hybridization and wash conditions as set forth above.

The present invention further provides for a *Vezf1* promoter sequence, as contained in a 20 kb genomic fragment (FIGURE 16), isolated by screening a 129 mouse genomic lambda phage library. The genomic fragment contains approximately 15 kb of genomic sequences upstream of the coding ATG, the first and second exons and the first intron of the *Vezf1* gene. The promoter sequence contained in this fragment may be identified using standard techniques. The present invention further provides for the human *Vezf1* promoter, which may be identified by hybridization to the foregoing mouse 20 kb genomic fragment (as contained in *Vezf1.N*, see below) under stringent hybridization and wash conditions (hybridization in 50 percent formamide, 5x SSPE, 2x Denhardt's, 0.5 percent SDS, 100 μg/ml salmon sperm DNA, at 37°C; and washes in 0.2 x SSC, 0.1 percent SDS at 50°C).

Two purified and isolated nucleic acids, together comprising *Vezf1* cDNA, termed *mVezf1.1* (3-1444) and *mVezf1.2*(1394-2820), containing nucleic acid residues 3-1444 (in a 1.444 kb EcoRI-EcoRI cDNA fragment) and 1394-2820 (in a 1.429 kb EcoRI-EcoRI cDNA fragment) of murine *Vezf1* cDNA, respectively, in pBluescript II SK (grown in DH5α) and a nucleic acid, termed *mVezf1.N*, containing the forementioned 20 kb genomic NotI-NotI fragment in pBluescript II SK (grown in DH5α), have been deposited under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC), having an address of 12301 Parklawn

Drive, Rockville, Maryland 20852 on ____________, 1998 and assigned accession nos. __________, __________ and _____________, respectively.

The present invention also provides for nucleic acid molecules which hybridize to a *Vezf1* gene, including complementary DNA and RNA molecules (referred to as "corresponding" to the *Vezf1* gene), including mRNA transcripts of the *Vezf1* gene as well as nucleic acid molecules which are "anti-sense", that is to say, complementary to a mRNA transcript of the *Vezf1* gene. Preferably, the conditions for hybridization and washing are as follows: hybridization in 50 percent formamide, 5x SSC, 2x Denhardt's, 0.5 percent SDS, 100 μg/ml salmon sperm DNA at 42°C, and washes is 0.2 SSC, 0.1 percent SDS at 50°C.

The present invention still further provides for purified and isolated *Vezf1* proteins. The term "*Vezf1* protein", as used herein, includes murine *Vezf1* protein, having an amino acid sequence as set forth in FIGURE 1 (SEQ ID NO:2), the human DB1 protein having an amino acid sequence as set forth in FIGURE 2 (SEQ ID NO:4), and proteins which are at least 90 percent, and preferably 95 percent, homologous to mouse *Vezf1* protein as determined by MACVECTOR and the Blast Search Algorithm.

The *Vezf1* gene or a corresponding cDNA or RNA may be incorporated into any suitable cloning or expression vector, operably linked to appropriate control elements (*e.g.*, promoter/ enhancer elements, ribosomal binding sites, polyadenylation sites, termination sites, etc.). Examples of such vectors include, but are not limited to, herpes simplex viral based vectors such as pHSV1 (Geller et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:8950-8954); retroviral vectors such as MFG (Jaffee et al., 1993, Cancer Res. 53:2221-2226), and in particular Moloney retroviral vectors such as LN, LNSX, LNCX, LXSN (Miller and Rosman, 1989, Biotechniques

7:980-989); vaccinia viral vectors such as MVA (Sutter and Moss, 1992, Proc. Natl. Acad. Sci. U.S.A. 89:10847-10851); adenovirus vectors such as pJM17 (Ali et al., 1994, Gene Therapy 1:367-384; Berker, 1988, Biotechniques 6:616-624; Wand and Finer, 1996, Nature Medicine 2:714-716); adeno-associated virus vectors such as AAV/neo (Mura-Cacho et al., 1992, J. Immunother. 11:231-237); lentivirus vectors (Zufferey et al., 1997, Nature Biotechnology 15:871-875); and plasmid vectors such as pCDNA3 and pCDNA1 (InVitrogen), pET 11a, pET3a, pET11d, pET3d, pET22d, pET12a and pET28a (Novagen); plasmid AH5 (which contains the SV40 origin and the adenovirus major late promoter), pRC/CMV (InVitrogen), pCMU II (Paabo et al., 1986, EMBO J. 5:1921-1927), pZipNeo SV (Cepko et al., 1984, Cell 37:1053-1062), pSRα (DNAX, Palo Alto, CA) and pBK-CMV, pSPTg.T2FpAXK and pSPTg.2FXK (Schaleger et al., 1997, Proc. Natl. Acad. Sci. U.S.A. 94:3058-3063).

In specific, nonlimiting embodiments of the invention, a *Vezf1* gene or a corresponding cDNA or RNA may be incorporated into an expression vector as part of a dicistronic expression cassette together with a second gene. For example, such a gene may be a reporter gene (*e.g.*, human placental alkaline phosphatase, luciferase, β-galactosidase, etc.).

In other specific, nonlimiting embodiments of the invention, an expression vector may comprise a *Vezf1* gene (or a corresponding cDNA or RNA) operably linked to a heterologous promoter (*i.e.*, a promoter not found, in nature, linked to the *Vezf1* gene) known or believed to be selectively expressed in vascular endothelial cells, including, but not limited to, the *Tie2* promoter enhancer (Schlaeger et al., 1997, Proc Natl. Acad. Sci. U.S.A. 94:3058-3063) and the *flk-1* promoter/enhancer (Rönicke et al., 1996, Circ. Res. 79:277-285). In alternative embodiments, the heterologous promoter may be an inducible promoter, such as the

metallothionine promoter.

The present invention further provides for antibody molecules which specifically bind to a *Vezf1* protein. According to the invention, a *Vezf1* protein, its fragments or other derivatives, or analogs thereof, may be used as an immunogen to generate antibodies which immunospecifically bind such an immunogen. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. In specific embodiments, antibodies to mouse *Vezf1* protein or to human DB-1 protein are produced.

Various procedures known in the art may be used for the production of polyclonal antibodies which specifically bind to a *Vezf1* protein. In a particular embodiment, rabbit polyclonal antibodies to an epitope of a *Vezf1* protein having an amino acid sequence set forth in FIGURE 1 (SEQ ID NO:2) or FIGURE 2 (SEQ ID NO:4) may be obtained. For the production of antibody, various host animals can be immunized by injection with the native *Vezf1* protein, or a synthetic version, or derivative (e.g., fragment) thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete or incomplete) adjuvant, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

For preparation of monoclonal antibodies directed toward a *Vezf1* protein, any technique which provides for the production of antibody molecules by continuous cell lines in

culture may be used. Examples of such techniques include the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBVhybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for *Vezf1* together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce *Vezf1*-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246:12751281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for *Vezf-1* protein derivatives, or analogs.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(abl), fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(abl), fragment, the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, e.g. ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies which recognize a specific domain of a *Vezf1* protein, one may assay generated hybridomas for a product which binds to a *Vezf1* fragment containing such domain. For selection of an antibody that specifically binds a first *Vezf1* homolog but which does not specifically bind a different *Vezf1* homolog, one can select on the basis of positive binding to the first *Vezf1* homolog and a lack of binding to the second *Vezf1* homolog.

5.2 DIAGNOSTIC METHODS

The present invention provides for a method of diagnosing a vascular disorder in a subject, whereby the presence, amount, and/or molecular characteristics of the *Vezf1* gene, its corresponding mRNA or a cDNA thereof, or its gene product are determined and compared to normal values. Differences in the molecular characteristics of the *Vefz1* gene or its expression would support a diagnosis of a vascular disorder or would indicate that the subject is capable of genetically transmitting such a disorder to its progeny.

The term "vascular disorder", as used herein, includes disorders which primarily

arise in the vasculature as well as conditions which primarily involve other tissues or organs but in which angiogenesis is either undesirable (e.g., neoplasms and rheumatoid arthritis) or advantageous (e.g., in ischemic conditions and wound healing).

In particular, the present invention provides for a method of diagnosing a vascular disorder in a subject, comprising measuring the amount of a *Vezf1* gene product, where the gene product is selected from the group consisting of RNA and protein, in a test sample taken from the subject and comparing that amount to the amount of *Vezf1* gene product in a matched control sample, wherein a difference in the amount of *Vezf1* gene product in the test sample and the control sample correlates with the presence of a vascular disorder in the subject.

In particular, the present invention provides for a method of diagnosing a disorder involving angiogenesis in a subject, comprising measuring the amount of a *Vezf1* gene product, where the gene product is selected from the group consisting of RNA and protein, in a test sample taken from the subject and comparing that amount to the amount of *Vezf1* gene product in a matched control sample, wherein a difference in the amount of *Vezf1* gene product in the test sample and the control sample correlates with the presence of a disorder involving angiogenesis in the subject. An increased amount of *Vezf1* gene product in the test sample positively correlates with increased angiogenesis in the disorder, and a decreased amount of *Vezf1* gene product in the test sample positively correlates with decreased angiogenesis in the disorder.

The present invention also provides for a method of diagnosing a heritable vascular disorder in a subject (including the ability to have an offspring suffering from a vascular disorder which is not apparent in the parental phenotype), comprising characterizing a *Vezf1* gene in the subject and comparing the characteristics of the gene to the normal *Vezf1* gene, where a

difference in characteristics of the *Vezf1* gene in the subject and the normal *Vezf1* gene correlates with the presence of a heritable vascular disorder in the subject.

For example, the characteristics of a *Vezf1* gene in a subject may be evaluated by analyzing the DNA of the subject. For example, a sample of DNA obtained from the subject may be prepared and then subjected to Southern analysis, using, as a hybridization probe, a *Vezf1* gene or cDNA or portion thereof (hereafter, "a *Vezf1* probe"). Using such a technique, the DNA of the subject may be cleaved by one or more restriction endonucleases, the resulting fragments may be resolved electrophoretically, e.g. in an agarose gel, and then transferred to a membrane (e.g. nitrocellulose) and hybridized to a *Vezf1* probe. A control DNA sample, subjected to the same treatments, may be run in parallel as a control. A different pattern of restriction fragments in the DNA sample of the subject relative to the pattern observed in control DNA may indicate an abnormality in the *Vezf1* gene in the subject.

In specific, nonlimiting examples, the *Vezf1* probe may be a nucleic acid having a sequence as set forth in FIGURE 1 (SEQ ID NO:1) or FIGURE 2 (SEQ ID NO:3), or a subsequence thereof, wherein the subsequence is at least 10 nucleotides long, and more preferably at least 50 nucleotides long. The *Vezf1* probe may be detectably labeled, for example, with a radioisotope or a detectable molecule, such as biotin.

Similarly, the expression of *Vezf1* RNA in a subject may be evaluated. The size and amount of the *Vezf1* mRNA transcript in a cell or tissue sample of a subject may be determined, for example, using Northern analysis, wherein RNA prepared from the sample is electrophoretically separated in the presence of formamide, transferred to a membrane (e.g. nitrocellulose), and then hybridized to a *Vezf1* probe as described above. Where the size or

amount of *Vezf1* RNA in the sample differs relative to the size or amount of *Vezf1* RNA in a matched control sample, a disorder in *Vezf1* expression, or a disorder associated with abnormal *Vezf1* expression, is indicated. In humans, the normal size of the *Vezf1* (also known as DB-1) mRNA transcript is appoximately between 4.0 and 4.8 kb.

Further, the expression of *Vezf1* protein in a subject may be evaluated. The size and amount of *Vezf1* protein in a cell or tissue sample of a subject may be determined, for example, by Western blot analysis, wherein protein prepared from the sample is electrophoretically separated (e.g., in a polyacrylamide gel), transferred to a membrane (e.g., nitrocellulose) and then bound to anti-*Vezf1* antibody. To quantitate the amount of protein present, an ELISA assay may be used. Where the size or amount of *Vezf1* protein in the sample differs relative to the size or amount of *Vezf1* protein in a matched control sample, a disorder in *Vezf1* expression, or a disorder associated with abnormal *Vezf1* expression, is indicated. The apparent normal size of the human *Vezf-1* protein, DB-1, is about 56-80 kDa..

Still further, *Vezf1* expression at the RNA or protein level may be used to detect or measure the presence or amount of vascular endothelial cells in a cell or tissue sample of a subject.

The detection of primary genetic defects in the *Vezf1* gene (as indicated, for example, by restriction fragment length polymorphism or an abnormal size of mRNA transcript) or abnormalities in the level or timing of *Vezf1* expression bear a positive correlation with a diagnosis of a heritable vascular disorder. The propensity of such a subject to transmit such a disorder to its offspring would be supported.

Where the *Vezf1* gene itself in a subject is normal, and the control of its

expression is normal, an abnormally high level of *Vezf1* mRNA or protein may indicate the presence of a disease or disorder which increases proliferation of vascular endothelial cells. Examples of such conditions include, but are not limited to, damage to the existing vascular endothelium by trauma (including intrinsic and iatrogenic) or a disease process, such as atherosclerosis, neoplasia, or the generation of new tissue, as in wound healing or pannus formation in rheumatoid arthritis, or other disorders which result in the proliferation of blood vessels, such as malignant endothelial angiomatosis. As the metastatic potential of a malignant tumor may correlate with its vascularity, a determination of the level of *Vezf1* mRNA or protein expression in a tumor sample may have prognostic value, where high levels may bear a positive correlation with increased metastatic potential.

Analogously, *Vezf1* levels may be decreased in necrotic cells or tissues, post-mitotic or resting endothelial cells, such that a determination that the *Vezf1* level in a tissue or cell is decreased relative to normal tissues may indicate that the cell or tissue is necrotic or in a resting state. Further, a decrease in *Vezf1* level in a subject may indicate a regression in a condition associated with an increase in *Vezf1* expression; as an example, the level of *Vezf1* may fall in a tumor when the tumor regresses.

5.3 MODEL SYSTEMS

The present invention provides for non-human animal model systems which may be used to evaluate the effects of increasing, decreasing, or altering the temporal expression pattern of *Vezf1* expression. Such animals may carry, as a transgene, an exogenously introduced *Vezf1* gene or a *Vezf1* cDNA, in some or all of their cells, and/or may have a structural alteration in one or more of their endogenous *Vezf1* genes. Such an alteration may be a "knock-out" of one or more endogenous *Vezf1* gene. The exogenous *Vezf1* gene or cDNA may be present in some or all cells of the animal.

In specific, nonlimting embodiments of the invention, a *Vezf1* cDNA exogenously introduced into a non-human animal may have a nucleic acid sequence as set forth in FIGURE 1 (SEQ ID NO:1) or FIGURE 2 (SEQ ID NO:3), and may be operably linked to a promoter element. The promoter element may be a *Vezf1* promoter, another promoter selectively active in vascular endothelial cells (such as the *Tie2* promoter), a promoter active during substantially the same developmental stages as the *Vezf1* promoter, or an inducible promoter (such as the metallothionine or tetracycline promoter) or a promoter which directs widespread or ubiquitous expression (such as a human cytomegalovirus promoter or a retroviral LTR promoter).

Transgenic animals carrying a *Vezf1* gene, or a portion or mutation thereof, may be created by standard techniques, including, but not limited to, techniques described in United States Patent No. 4,736,866, PCT publication WO82/04443, PCT publication WO88/00239. Such animals may also be produced by infection with vectors carrying *Vezf1* encoding nucleic acid in expressible form, or by inoculation with naked DNA encoding a *Vezf1* protein or mutant or subfragment thereof. Animals in which the endogenous *Vezf1* gene or its control elements

have been altered by homologous recombination may be produced using techniques as set forth in United States Patent No. 5,464,764 or Bradley, 1991, Current Op. Biotechnol. 2:823-829. The present invention also contemplates animals in which one or more endogenous gene has been "knocked out" and an exogenous *Vezf1* gene or cDNA has been introduced.

Non-limiting examples of animals which may serve as non-human animal model systems include mice, rats, rabbits, chickens, sheep, goats, cows, pigs, and non-human primates.

For example, non-human animals may be produced in which extra copies of the *Vezf1* gene or cDNA have been introduced, or a *Vezf1* gene under a strong promoter has been created or introduced into the animal, such that the level of *Vezf1* protein in the animal, or in a subset of cells of the animal, has been increased relative to normal levels. Preferably the increase is by at least 25 percent. Such animals may be used to study the effects of increased *Vezf1* levels, or may be used to identify compounds which act as antagonists of *Vezf1* activity.

Alternatively, non-human animals may be produced which have lower than normal levels of *Vezf1* expression in some or all cells, by virtue of a defect introduced in one or more alleles of the endogenous *Vezf1* gene and/or its control elements. For example, but not by way of limitation, one or both alleles of the endogenous *Vezf1* gene in the animal may be eliminated or mutated by gene targeting techniques. Alternatively, an additional *Vezf1* gene with altered structure may be introduced which competes with endogenous *Vezf1* and thereby inhibits native *Vezf1* activity. Examples of mutated forms of the *Vezf1* gene include variant genes which produce a truncated *Vezf1* protein, for example,a *Vezf1* protein lacking one or more zinc finger motifs, or a *Vezf1* protein which eliminates non-zinc-finger related amino acid sequence (see, for nonlimiting examples, the constructs shown in FIGURE 17).

5.4 METHODS OF TREATMENT

The present invention provides for methods of treating a vascular disorder comprising altering the activity of *Vezf1* in the subject.

In certain embodiments of the invention, the vascular disorder is associated with undesirable angiogenesis, in which case it is desirable to decrease *Vezf1* activity in a subject or in a particular tissue or cell population of the subject. Such a decrease may be achieved by either inhibiting the expression of endogenous *Vezf1* genes, for example, by the administration of an antisense RNA or ribozyme or an inhibitor of the *Vezf1* promoter, or inhibiting the action of *Vezf1* protein, for example, by administering an antagonist of *Vezf1* (which may be identified using an animal model system described above) or by administering an anti-*Vezf1* antibody (or a fragment or derivative thereof). Conditions which may benefit from such treatment include, but are not limited to, neoplasms (where the blood supply to the neoplasm may be compromised), atherosclerosis, post-angioplasty restenosis, diabetic retinopathy, rheumatoid arthritis, hemangiomas, psoriasis, duodenal ulcers, and vascular malformations (Folkman, 1995, Nature Med. 1:27-31).

In other embodiments, the vascular disorder is associated with insufficient angiogenesis, or the primary disorder is one in which increased angiogenesis is desirable. Under such circumstances, the activity of *Vezf1* in a subject, or in a particular tissue or cell population of the subject, may be increased. Such an increase may be achieved, for example, by increasing the transcription of *Vezf1* by administering an agent which stimulates the *Vezf1* promoter, or by increasing the number of *Vezf1* genes (e.g. by gene therapy, for example using a retroviral or adenovirus vector comprising a *Vezf1* gene or cDNA operably linked to a promoter element).

Alternatively, *Vezf1* protein may be administered to the subject, or an agonist of *Vezf1* activity may be administered. Conditions which may benefit from such treatment include ischemic conditions (associated, for example, with myocardial, brain or peripheral vascular ischemia), wound healing, tissue grafting (including transplant) and conditions involving endothelial cell growth/proliferation, for example after coronary angioplasty, stenting or related procedures, re-endothelialization of arterial grafts, and endothelial regeneration in A-V shunts, e.g. in renal dialysis patients.

In particular embodiments, the present invention provides for a method of increasing angiogenesis in a tissue of a subject in need of such treatment, comprising increasing the amount of *Vezf1* activity in the tissue. Nonlimiting examples of such tissues include myocardial tissue, brain tissue, wounded tissue, and grafted tissue.

In alternative embodiments, the present invention provides for a method of decreasing angiogenesis in a tissue of a subject in need of such treatment, comprising decreasing the amount of *Vezf1* activity in the tissue. In a specific nonlimiting embodiment, the tissue is a neoplasm, and in particular a malignant neoplasm.

Administration of the foregoing agents may be local or systemic, using a suitable pharmaceutical carrier. Other compounds which aid in the uptake or stability of these agents, or which have beneficial pharmaceutical activity, may also be included in the formulations of the invention.

6. EXAMPLE: PREPARATION OF AN EMBRYONAL STEM CELL CLONE HAVING A RETROVIRAL INSERTION IN *Vezf1*

6.1. MATERIALS AND METHODS

Retroviral vectors and cells. Retroviral entrapment vectors containing a human alkaline phosphatase ("AP") reporter gene, namely PT-AP (which requires insertion in the proximity of a cellular promoter for AP expression, and hence is referred to as a "promoter trap vector"), GT-AP (which contains a splice acceptor site 5' of the AP gene which can combine with a splice donor site from cellular RNA, and hence is referred to as a "gene trap vector") and PT-IRES-AP (which contains an internal ribosomal entry site upstream of the AP gene, and which can form a bicistronic transcript with a cellular RNA), and $\Psi 2$ packaging lines stably producing the retroviral vectors, were as depicted in FIGURE 18. The ES cell line R1 (Nagy et al., 1993, Proc. Natl. Acad. Sci. U.S.A. 90:824-828) was obtained at passage 8 from A. Nagy, Toronto, Canada, and aliquots were prepared and frozen at passage 10. The R1 cells were maintained in ES cell culture medium (high glucose Dulbecco's Modified Eagles Medium ("DMEM") supplemented with 15 percent heat-inactivated bovine serum (HyClone); 20 mM HEPES, pH 7.3, 20 mM non-essential amino acids (GIBCO), β-mercaptoethanol (Sigma), penicillin and streptomycin, 500 U/ml LIF (GIBCO)). The cells were grown in gelatinized dishes on γ-irradiated embryonic fibroblasts as feeder cells that were prepared from day 13 IL-6 (-/-) embryos obtained from F. Constantini, Columbia University.

Infection of ES cells with retroviral entrapment vectors.

Infection by co-cultivation with virus producing cells. ψ2 producer clones (5 x 10^6 cells per 10 cm dish) stably transfected with the retroviral entrapment construct PT-AP were treated for 3 hours with 10 μg/ml mitomycin C (Boehringer), washed three times with PBS, trypsinized and plated onto 10 cm dishes in ES cell culture medium supplemented with 4 μg/ml polybrene (Sigma). After attachment, about 1 x 10^6 R1 cells and embryonic fibroblast feeder cells were plated onto the same dish. After 48 hours co-cultivation, the ES cells were trypsinized, and infected clones were selected for 7 days by plating them in ES cell medium supplemented with 350 μg/ml G418 (GIBCO).

Infection with virus supernatant. R1 cells, plated the preceding day at a density of 1 x 10^6 cells per 10 cm dish in ES cell medium supplemented with polybrene (4μg/ml), were infected by adding 2 ml of freshly harvested and filtered (0.4 μ filters) supernatant from Ψ2 clones producing GT-AP or PT-IRES-AP virus (multiplicity of infection ("MOI") between 0.01-0.1). Four hours later, 8 ml of ES cell medium was added. Thirty-six hours after infection, G418 selection medium was applied to the cells. Single g418-resistant colonies were isolated seven days later under a dissection microscope, trypsinized and divided into two parallel 96-well plates. Cells in one 96-well dish (the master plate) were frozen down, whereas cells from its parallel plate were expanded into 24-well dishes for further analysis.

Differentiation of ES cells into embryoid bodies. Individual ES cell clones, grown to 30-50 percent confluency in 24-wekk dishes, were pooled in groups of 6 to 8 and induced to spontaneously differentiate into embryoid bodies essentially as described in Doetschman et al., 1985, J. Embryol. Exp. Morph. 87:27-45 and Lindenbaum and Grosveld,

1990, Genes Dev. 4:2075-2085. Cells were plated in 6 cm gelatin-coated dishes in the absence of feeder cells in ES cell medium without LIF. After two days in culture, small ES cell clumps were lifted off the plates by gentle trypsinization and transferred into suspension culture (DMEM supplemented with 10 percent heat-inactivated fetal bovine serum; 20 mM HEPES, pH 7.3; penicillin and streptomycin) in 6 cm petri dishes (Labtech, Nunc). After four days, "simple embryoid bodies ("EBs") had formed, consisting of an outer layer of endodermal cells, a basal lamina and an inner layer of columnar, ectodermal cells (Robertson, 1987, in *Teratocarcinomas and embryonic stem cells: a practical approach*, Robertson, ed., IRL Press, Oxford, England, pp.71-112). The simple embryoid bodies were kept for another six days in suspension culture, where they further differentiated into complex "cystic embryoid bodies" containing visible cavitation, pockets of primitive blood islands, and rhythmically contracted cardiomyocytes. In parallel, day 4 simple embryoid bodies were re-plated on gelatinized tissue culture plates, where they attached within 24 hours. Outgrowth of differentiated cells with various phenotypes, including fibroblasts, endothelial-like cells, neurons, contracting cardiomyocytes, differentiated cell types with an uncharacterized phenotype, as well as pockets of undifferentiated cells, was observed during the next six days. Aliquots of embryoid bodies at day 4, day 7 and day 10 in suspension culture were harvested, fixed and histochemically stained for human placental alkaline phosphatase activity in the presence of the inhibitor levamisole (.24 mg/ml) as described in Fields-Berry et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89:693-697. To inactivate endogenous mouse AP activity, ES cells and embryoid bodies were heat-treated for 30 minutes at 70°C. Day-6 cultures of re-plated embryoid bodies were examined for AP activity as well. Once positive pools were identified, individual clones from these pools were analyzed for AP activity in the

form of undifferentiated ES cells, upon embryoid body formation, and in cultures of re-plated embryoid bodies. Only those ES cell clones that displayed reproducible AP expression in at least 50 percent of all embryoid bodies present in each culture sample were scored as positive. Positive ES cell clones were re-tested for their AP expression pattern, expanded, and frozen in aliquots for further analysis.

6.2. RESULTS

Developmentally regulated genes were sought to be identified using retroviral entrapment vectors, as depicted in FIGURE 3. Expression of the AP reporter gene in ES cells with entrapment vector insertions during *in vitro* differentiation was characteristic and highly reproducible for each individual clone, examples of which are illustrated in FIGURE 4A-X. AP staining was visible as a dark purple precipitate that was easily distinguished from the faint pink background in embryoid body cultures derived from uninfected R1 control cells (FIGURE 4A,B). Clone PT-AP #IVE3-8 showed weak expression in undifferentiated ES cells, widespread and strong expression in day-4, and weaker, more restricted expression in day-7 and day-10 EBs (FIGURE 4C,D). Clone GT-AP #7-65 displayed spotty AP staining exclusively during embryoid body formation, increasing in intensity between day-4 and day-10 cultures (FIGURE 4E,F). PT-IRES-AP clones #1-13 and #9-G7 showed spotty expression in embryoid bodies, varying in intensity between day-4 and day-10 cultures (#1-13, FIGURE 4G,H; #9-G7, FIGURE 4W,X), and restricted expression in re-plated embryoid bodies. In clones PT-IRES-AP #1-58 and #2-86, strong and patchy AP expression was restricted to day-7 and day-10 embryoid body cultures (FIGURE 4I,J and FIGURE 4K,L, respectively). PT-IRES-AP clone #2-51 showed spotty expression in undifferentiated ES cells and day-7 and day-10 embryoid bodies, but not in day -4

embryoid bodies or re-plated embryoid bodies (FIGURE 4M,N). Clone PT-IRES-AP #6-33 displayed spotty AP expression restricted to embryoid bodies, increasing in intensity from day-7 to day-10 cultures (FIGURE 4Q,R). Finally, PT-IRES-AP clones #4-45, #7-26, and #9E5 stained positive for the AP reporter gene in all stages of *in vitro* differentiation, including re-plated embryoid bodies, and they displayed either widespread (#7-26, FIGURE 4S,T) or patchy AP expression (#4-45, FIGURE 4O,P; #9E5, FIGURE 4U,V). Clone PT-IRES-AP #1-13 was chosen for further study.

7. EXAMPLE: PROMOTER TRAP INSERTION INTO *VEZF1*

7.1. MATERIALS AND METHODS

Molecular cloning of #1-13 retroviral flanking genomic DNA. Genomic DNA flanking the #1-13 retroviral insertion was cloned by *supF* complementation (Reik et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:1141-1145; Soriano et al., 1987, Genes Dev. 1:366-375). A recombinant DNA library was constructed by ligating EcoR1-cleaved genomic DNA from the #1-13 ES cell clone to the EcoRI site of λZAP vector DNA (Stratagene). The ligation mix was packaged *in vitro* using Gigapack II Gold packaging extract (Stratagene) and titered on *E. coli* VCS257 bacteria. About 2 x 10^6 plaques were used to infect the *supF*$^-$ strain *E. coli* MC1060. About two percent (4 out of 200) plaques that grew on MC1060 bacteria contained the proviral DNA insert, as shown by plaque hybridization with a *neo*- or AP-specific probe. pBluescript SK phagemids Stratagene containing the cloned insert were rescued from λZAP by *in vivo* excision, thereby producing a 0.7 kb #1-13 3' EcoRI flanking genomic DNA region probe.

Characterization of cDNAs. cDNAs corresponding to the #1-13 insertion were

isolated using the flanking region probe to screen a day-7.5 mouse embryonic cDNA library (provided by J. Gerhart). Sequencing was performed using a commercial sequencing kit (Sequenase Version 2.0, USB) and synthetic oligonucleotide primers specific for the U3 region (5' GAG TGA TTG ACT ACC C 3'; SEQ ID NO:5) or U5 region (5' GCT AGC TTG CCA AAC C 3'; SEQ ID NO:6) of the Mo-MLV LTRs and T3 or T7 primers, respectively. Two cDNA clones, termed 10-1 and 10-2, were isolated and sequenced as above. Sequencing homology analysis was performed using the BLAST comparison algorithm (Netscape Navigator, 3.01). Both cDNA clones were found to have significant homology with human DB1 cDNA (Koyano-Nakagawa et al., 1994, Mol. Cell. Biol. 14:5099-5107). Full length cDNA was obtained by RT-PCR of total RNA from day 7 embryoid bodies. The first strand DNA was synthesized in a 25 μl reaction containing 1 μg RNA, 2 pmol primer, 400 μM dNTPs, 10 mM DTT, 1x first strand buffer (Gibcol BRL) and 200 units of Superscript II (Gibcol BRL) at 42°C for one hour. The primer for the RT reaction was 5' TCATTGCTATATTGAGA 3'(SEQ ID NO:7) from *Vezf1* cDNA (nucleotides 1487-1503). The cDNA fragment of *Vezf1* was amplified in a 100 μl PCR reaction containing 5 μl of RT mixture, 20 pmol of primers, 200 μM dNTPs, 2 units of Vent (NEB), 1x Vent reaction buffer with 2 mM $MgSO_4$. The PCR primers used were, as 5' primer, EcoRI.DB1A: 5' GGAATTCCGGGGGGAGTGGGGAGGA 3' (SEQ ID NO:8) from human DB1 cDNA (nucleotides 3-20) and, as 3' primer, BamHI.12T7.2: 5' CGGGATCCCGTGATGGTGACAGGGTGTGCTA 3' (SEQ ID NO:9) from mouse *Vezf1* cDNA (nucleotides 1423-1443). The PCR cycles used were 94°C, 1 minute; 55°C, 1 minute; 72°C, 2 minutes, for 35 cycles. PCR fragments of *Vezf1* were subcloned into a pBluescript vector (Stratagene) and subjected to DNA sequencing.

ES cell cultures and embryoid bodies. Clone #1-13 ES cells prepared as described in the preceding section were maintained in an undifferentiated state by growing in gelatinized dishes on γ-irradiated mouse embryonic fibroblast feeder cells. ES cells were induced to differentiate into embryoid bodies *in vitro* by plating small ES cell clumps in suspension culture for 4-10 days. Day 4 embryoid bodies were re-plated on gelatinized tissue culture plates, and attached embryoid bodies outgrew to differentiate into different cell types, referred to as "re-plated embryoid bodies". ES cell culture medium and embryoid body medium were as described in the preceding section.

Generation of ES cell aggregation chimeras and transgenic lines. ES cells were used to generate germ-line chimeras by using the ES cell-diploid embryo aggregation method essentially as described in Nagy and Rossant, 1993, in *Gene targeting: a practical approach*, Joyner, Ed., Oxford University Press, NY, pp. 147-179.Briefly, ES cells were used to aggregate with 8-cell stage CD-1 mouse embryos, and aggregates were cultured at 37°C to develop into blastocysts or compacted morula. These were reintroduced into the uterus of day 2.5 pseudo-pregnant Swiss Webster foster mothers and allowed to develop to term. Chimeric males with strong ES cell contribution were identified by their agouti coat color and eye pigmentation. To test germline transmission, chimeric males were bred with CD-1 females, and agouti F1 offspring were examined for the presence of entrapment vector DNA in their tail DNAs. Inbred mice heterozygous for the insertion were generated by crossing with 129/Sv females. Homozygous offspring were obtained by intercross of F1 heterozygous parents. The genotypes of these animals were determined by Southern blot analysis on genomic DNA, using a #1-13 flanking genomic probe .

AP staining in whole-mount embryos and histological sections. Staged embryos were dissected out of the CD-1 females that were mated with heterozygous males carrying the #1-13 retroviral insertion. Mouse embryos and extraembryonic membranes from E7.5 to E13.5 were subjected to AP staining with the BM purple BCIP/NBT substrate (Boehringer Mannheim) in the presence of levamisole (Fields-Berry et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89:693-697). AP stained whole-mount embryos were embedded in paraffin, and sectioned by conventional methods (Hogan et al., 1994, *Manipulating the mouse embryo: a laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Whole mount and sectioned embryos were photographed under a Nikon SMZ-U stereomicroscope with a digital camera.

RNA *in situ* hybridization. Clone 10-1 of the *Vezf1* cDNA, corresponding to nucleotides 1397 through 2899, was used as a template for both sense and antisense probe synthesis by RNA *in vitro* transcription. *In situ* hybridization to sectioned embryos was performed as described in Sassoon and Rosenthal, 1993, Meth. Enzymol. 225:384-404. Birefly, embryoid bodies or staged mouse embryos fixed in 4 percent formaldehyde were embedded in paraffin at 60°C following a series of dehydrating washed in ethanol and a final 30 min wash in xylene. Material sectioned at 6µm was de-paraffinized and digested with proteinase K followed by a 16 hour hybridization with ^{35}S-radiolabeled antisense RNA probes (final probe concentration 75 dpm/ml in hybridization buffer). Post-hybridization washes of increasing stringency were included to reduce background. Sense control probes were synthesized for*Brachyury* and *Fgf-5*. Slides were dipped in Kodak NBT-2 emulsion, dried overnight and exposed for 5 days to 2 weeks at 4°C. Material was counter-stained with toludine blue, unless it

was stained before as whole mount for AP, and photographs were taken using a Lica Leitz DMRB Microscope.

RNA isolation and Northern hybridization analysis. Total RNA was prepared from tissues and cell lines by using a modification of the guanidinium-CsCl method described in Chirgwin et al., 1979, Biochemistry 18:5294-5299. Tissues were homogenized in guanidinium buffer (4M guanidinium thiocyanate, 25 mM Na citrate, pH 7.0, 0.5 percent sarcosyl, 0.1 M β mercaptoethanol). Cultured cells and embryoid bodies were washed in ice cold PBS and lysed in guanidinium buffer, and the viscosity of the lysate was reduced by shearing through a 21-gauge needle. RNA was pelleted through a CsCl step gradient (6.2 M CsCl, 0.1 M EDTA, pH 8.0) by centrifugation for 18 hours at 35,000 rpm at 15°C. RNA pellets were washed with 80 percent ethanol/TE (where "TE" is 10 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0), dissolved in TE, and precipitated by adding 0.1 volumes of 3M Na acetate and 2.5 volumes of ethanol. 20 μg samples of RNA were electrophoresed on 1 percent agarose-formaldehyde gels using standard procedures. Gels were transferred to nitrocellulose membranes (Optitran, Schleicher & Schuell) in 20X SSC. The filters were hybridized with 1 x 10^7 cpm of random hexamer-primed, [α-^{32} P] dCTP-labeled fragment of the *Vezf1* cDNA (nucleotides 3-776) at 42° C in 50 percent formamide. The filters were washed at 50° C in 0.2X SSPE (36 mM NaCl, 2 mM $NaPO_4$, pH 7.7, 0.2 mM EDTA), 0.1 percent SDS and exposed for 1-2 days. To control for RNA loading, filters were stripped and hybridized with a [α-^{32} P] dCTP-labeled mouse GADPH cDNA plasmid.

Southern analysis of genomic DNA and "zoo blot" analysis. High molecular weight DNA from cultured cells and tail biopsies was prepared as described in Laird et al., 1991, Nucleic Acids Res. 19:4293. Agarose gel electrophoresis and Southern analysis were performed

as described in Stuhlmann et al., 1990, J. Virol. 64:5783-5796. For Southern analysis of "zoo blots", high molecular weight DNA was prepared from various vertebrate and invertebrate species. 20 μg DNA of mouse or human DNA, and equivalent amounts of DNA from other species reflecting their genome complexity, were digested with BamHI, EcoRI or HindIII, respectively. Digested DNA was separated on 0.8 percent agarose gels and transferred to nitrocellulose membranes (Optitran, Schleicher & Schuell). The filters were hybridized to 2 x 10^7 cpm of a [α-^{32} P] dCTP-labeled fragment of the *Vezf1* cDNA, corresponding to nucleotides 697-1480, including 3 ½ of the six zinc fingers, under reduced stringency conditions (35° C in 50 percent formamide, 5X SSC, 5X Denhardt's, 0.5 percent SDS, and 100 μg/ml denatured salmon sperm DNA). Blots were washed at increasing stringency, with the final washes at 65° C in 0.2X SSC, 0.1 percent SDS for 30 minutes (twice), and exposed for 2-4 days.

Chromosomal mapping. A DNA panel from the Jackson Laboratory community resource (C57Bl/6J x *M. spretus*)F1 x *M. spretus* interspecific backcross (Rowe et al., 1994, Mamm. Genome 5:253-274) was used to map the *Vezf1* locus using PCR techniques. Single strand conformational polymorphism (SSCP) between the two parental strains C57Bl/6J and *M. spretus* was detected using a pair of primers that span an intron in the 3' flanking genomic DNA. The PCR primers were 5' GGAGGTGTTTGTACACATGCTCTG 3' (Map 3A; SEQ ID NO:10) and 5' GCTGTGGATGGAGCAGAATCCAGT 3' (Map 3B, SEQ ID NO:11). A 25 μl PCR reaction containing 50 ng DNA template, 1X PCR buffer, 50 μM dNTPs, 0.5 mM Mg^{2+}, 10 pM of each primer, 1 unit of Taq polymerase (NEB BioLabs) and 10 μCi [α-^{32} P] dCTP per eight reactions was subjected to 35 cycles of PCR having the conditions 94° C for 1 minute, 55° C for 1 minute, and 72° C for 2 minutes. The PCR products were resolved on a 6 percent non-

denatured polyacrylamide gel (Spinardi et al., 1991,) at 30 W constant power at 4° C for about 3 hours. Each sample was genotyped for the presence of the *M. spretus* allele and the results were transmitted back to the database of the Jackson Laboratory for comparison with the existing map.

6.2. <u>RESULTS</u>

Identification of the PT1-13 clone during ES cell differentiation. The #1-13 ES cell clone was identified and isolated based on AP reporter gene expression during R1 ES cell differentiation into EBs *in vitro* (see preceding section and FIGURE 5A-D). The #1-13 ES cells tested were either (i) ES cells which had been induced spontaneously to form embryoid bodies in suspension culture, or (ii) re-plated day-4 embryoid bodies in gelatin-coated tissue culture dishes. Aliquots of embryoid bodies in suspension culture at day-4, day-7 and day-10, or re-plated day-4 embryoid bodies were harvested and assayed for AP expression. Spotty purple staining was observed in day-4 simple embryoid bodies (as indicated by the arrow in FIGURE 5A) and even more appearent on the surface of transparent cystic bag-like structures in certain advanced day-4 embryoid bodies (as indicated by the arrowhead in FIGURE 5A). Stronger purple staining was observed around all day-7 cystic embryoid bodies (FIGURE 5B), but less staining was observed in day-10 embryoid bodies (FIGURE 5C). Strong purple staining was observed in groups of unidentified cells of the replated day-4 embryoid body monolayers in gelatin-coated dishes (FIGURE 5D). Therefore, AP reporter gene expression was detected in the simple embryoid bodies originating from ES clone #1-13, in which several early cell lineage marker genes such as Oct-3, Fgf-5, GATA-4 and Flk-1 are expressed.

Isolating proviral flanking genomic DNA. The retroviral IRES-PT-AP vector used to generate clone #1-13 ES cells contained a unique EcoRI restriction site located between

the AP gene and the PGK-neo cassette (see FIGURE 3). A 0.7 kb 3' EcoRI flanking genomic DNA fragment was cloned by *supF* complementation (see FIGURE 6A) and subjected to DNA sequencing. Southern analysis was performed (FIGURE 6B) using high molecular weight DNA from R1 ES cells, the #1-13 ES clone, and transgenic lines containing the #1-13 retroviral insertion, using the 0.7 kb fragment of flanking cellular DNA as a probe. In R1 ES cell DNA, only a 8.0 kb endogenous EcoRI fragment of the *Vezf1* gene was detected. Clone #1-13 ES cell DNA contained the 8.0 kb endogenous fragment as well as a 2.8 kb fragment which hybridized to the probe. In mice, wild-type (CD-1) DNA contained the 8.0 kb band, DNA from heterozygous mice exhibited bands representing the 8.0 kb endogenous fragment, and the 2.8 kb fragment corresponding to the retroviral insertion. DNA from mice homozygous for the #1-13 insertion lacked the endogenous 8.0 kb fragment. This data indicates that the 0.7 kb genomic fragment isolated by *supF* complementation is indeed derived from the endogenous gene containing the #1-13 retroviral insertion.

Isolation of *Vezf1* cDNA. The 0.7 kb flanking genomic DNA fragment was used as a probe to screen a E7.5 mouse embryo cDNA library. Two classes of overlapping cDNA, as contained in clones 10-1 and 10-2, were isolated. Both share significant homology to DB1. RT-PCR was used to obtain a full-length coding cDNA of *Vezf1* by using a 3' primer derived from clone 10-1 and a 5' primer from the human DB1 cDNA (see Materials and Methods, *supra*). A 3,640 bp full-length *Vezf1* cDNA was isolated using these two complementary approaches, and was subjected to DNA sequencing to obtain the nucleic acid and deduced amino acid sequences shown in FIGURE 1. This gene was named *Vezf1*, for "vascular endothelial zinc finger", based on its expression pattern and structural hallmark. An in-frame ATG codon is found in the

sequence at a position consistent with its use as an initiation codon, and a typical GC-rich sequence is found in proximity to the 5' end of the predicted coding sequence. From the deduced amino acid sequence, the predicted Mr of mouse *Vezf1* is approximately 56 kd. *Vezf1* contians six zinc finger domains of the Cys2/His2 type (FIGURE 1, underlined; similar to the zinc finger domains of DB1, Koyano-Nagakawa et al., 1994, Mol. Cell. Biol. 14:5099-5107), a glutamine stretch (indicated by asterisks in FIGURE 1), and periodically appearing proline residues in the carboxy-terminal 100 amino acids. When the deduced amino acid sequence of mouse *Vezf1* was aligned with and compared to the human DB1 sequence (Koyano-Nagakawa et al., 1994, Mol. Cell. Biol. 14:5099-5107), it was found that the two proteins share 98 percent sequence identity. Database searches have revealed that mouse *Vezf1* has 92 percent amino acid sequence identity over the last five zinc fingers with mouse Pur-1, a homolog of human Maz (ZF87) protein, which specifically binds to the *myc* promoter. Amino acid sequence alignment of residues 168-360 of mouse *Vezf1* (SEQ ID NO:12) and residues 275-469 of mouse Pur-1 (SEQ ID NO:13) is shown in FIGURE 7.

Retroviral insertion into the 3'UTR of the *Vezf1* gene. When the 0.7 kb retroviral-flanking genomic DNA fragment referred to above was sequenced and aligned with *Vezf1* cDNA, the retroviral insertion was found to be located in the 3' untranslated region (UTR) of the *Vezf1* gene (FIGURE 8).

Transgenic mouse lines carrying the #1-13 retroviral insertion. Clone #1-13 ES cells were used to generate complete mouse embryos by ES cell-morula aggregation techniques, and the embryos were allowed to develop to term to produce chimeric mice. Heterozygous mice carrying the retroviral insertion were obtained by breeding chimeric males

with wild type CD-1 or 129/Sv females. Homozygous mice were generated from intercrosses between heterozygous mice carrying the retroviral insertion. Genotypes of offspring were confirmed by Southern analysis of DNA obtained from tail biopsies. No obvious phenotypes were observed in either heterozygous or homozygous mice carrying the insertion, which may be explained by the observation that insertion had occurred in an untranslated region of the gene, resulting in incomplete disruption of its expression.

AP reporter and *Vezf1* gene expression.

Expression in hemangioblasts. AP expression was first detected in the extraembryonic mesoderm of the yolk sac as a ring-shaped stripe in whole-mount embryos at about E7.25 (FIGURE 9A). From histological sections of E7.25 embryos, restricted AP expression in visceral mesoderm of yolk sac, but not extraembryonic endoderm, was detected (FIGURE 10A). No detectable expression was observed in allantois or the embryo proper at this stage. *In situ* RNA hybridization using a riboprobe corresponding to nucleotides 1397 to 2820 in the cDNA (probe C in FIGURE 8) of sectioned E7.25 embryos showed restricted silver grains in the visceral extraembryonic mesoderm (FIGURE 11A). It therefore appears that AP expression faithfully represents *Vezf1* gene expression *in vivo.* AP and *Vezf1* RNA expression in the yolk sac appeared concomitantly with the beginning of blood island formation.

Expression during early vasculogenesis. As the neural plate formed (at about stage E7.5), strong AP expression expanded in the yolk sac of embryos, and AP expressing cells moved into the embryo proper. No AP staining in endocardial cells was detectable (FIGURE 9B), despite the fact that *flk-1* RNA expression has been observed at this stage (Yamaguchi et al., 1993, Development <u>118</u>:489-498). In sectioned E7.5 embryos, strong AP

expression was detected in the extraembryonic mesoderm, and no expression was observed in extraembryonic endoderm (FIGURE 10 E,F). Some weak AP expression was seen in embryonic mesoderm of the most anterior and posterior regions (FIGURE 10E,F). No AP expression was found in the ectoplacental core, allantois, embryonic ectoderm and endoderm (FIGURE 10E,F). At E8.5, very strong AP staining was detected in the whole embryo, strong expression was found in the allantois, and relatively weak expression was detected in yolk sac membranes (FIGURE 9C). In sections of E8.5 embryos, more specific AP expression was located in endothelial cells of dorsal aorta, putative angioblast cells in cephalic mesenchyme, endocardial tubes, and vitelline veins (FIGURE 12A,D). Careful examination of sections through E8.5 yolk sac membranes revealed AP expressing cells in yolk sac mesoderm lining the perimeter of blood islands. These yolk sac mesoderm cells form the vascular endothelium that surrounds the inner region of hematopoietic stem cells. No detectable AP expression in hematopoietic stem cells was observed (FIGURE 12H). *In situ* hybridization analysis of sections of E8.5 embryos showed intense areas of *Vezf1* expression in the cephalic mesenchyme, the labeled inner layer of endothelial cells in the dorsal aorta, and the endocardium (FIGURE 11C and 11E). This further confirms that AP expression accurately parallels that of *Vezf1*.

Expression during angiogenesis. In E9.5 embryos, AP staining was detected in the whole vascular plexus of the embryo proper and in yolk sac membranes from heterozygous embryos (FIGURE 9D). No detectable AP staining was observed in wild-type CD-1 embryos. Similar patterns of AP staining of vascular plexus were observed from E10.5 to E13.5, in which the expression of AP was observed to decrease over time (FIGURE 9E and 9F). In sections of E9.5 embryos, AP expression in head mesenchyme showed small clumps of

stained cells which may be putative angioblasts. Endothelial cells of the dorsal aorta and its derivatives, such as vessels in the branchial arches and intersomites, endothelial cells forming capillaries, and the endocardium showed strong AP staining (FIGURE 12E-H). The endothelial cell-specific AP expression was consistent with *Vezf1* gene expression during mouse embryonic angiogenesis (FIGURE 11E-J). In sections of E9.5 embryos, high levels of *Vezf1* expression were detected in the putative angioblasts in head mesenchyme, the meningeal plexus surround the brain and spinal cord (FIGURE 11E,F), the endocardium (FIGURE 11H), endothelial cells of the dorsal aorta (FIGURE 11F), intersomites (FIGURE 11G), the branchial arches, vitelline veins, and blood vessels in mesonephrons and lung and liver rudiments (FIGURE 11I,J). In sections of E13.5 embryos, *Vezf1* expression was observed in the endothelial cells of vascular plexi.

No detectable AP expression was observed in either erthroid precursors in the yolk sac, in nucleated blood cells from all stages of embryos tested, or in E13.5 fetal liver other than what appeared to be the endothelial lining of capillaries. The data therefore indicates that *Vezf1* expression is mainly confined to vascular endothelial cells and their precursors rather than hematopoietic cells.

***Vezf1* transcripts.** To determine the size, temporal and cell-type specific expression of *Vezf1* transcripts, Northern analysis was performed using RNA harvested from staged embryos, embryoid bodies, cell lines, and tissues from adult mice using a mouse *Vezf1* cDNA fragment extending from nucleotide 1 to 750 as a probe (probe A in FIGURE 8). Hybridization to a GADPH probe was used to standardize the RNA amounts and to establish the integrity of the RNA used (FIGURE 13A, 13B and 13C). A major 4.0 kb transcript and a low

abundance 1.0 kb transcript were detected in embryonic stages E8.5 - E13.5, R1 ES cells and embryoid bodies, and all mouse hematopoietic cell lines and endothelial cell lines tested (FIGURE 13A and 13B). ES cells had less *Vezf1* expression than embryoid bodies, and no significant changes in *Vezf1* gene expression were observed in different stages of embryoid bodies or in different mouse embryo stages. All mouse endothelial cell lines tested had the same transcripts as mouse embryos (FIGURE 13B). Among hematopoietic cell lines, T cell and B cells lines had more abundant *Vezf1* transcripts than a mouse erythroleukemia ("MEL") cell line or macrophages (FIGURE 13B), which is consistent with observed human DB1 expression in Jurkat Tcells (Koyanao-Nakagawa et al., 1994, Mol. Cell. Biol. 14:5099-5107). Human umbilical vein endothelial cells ("HUVEC") were found to have a major 4.0 kb transcript as well as a low abundance 4.5 kb transcript (FIGURE 13B), consistent with previously observed DB1 expression in HUVEC cells (Koyanao-Nakagawa et al., 1994, Mol. Cell. Biol. 14:5099-5107).

Three *Vezf1* transcripts, two abundant transcripts of 4.0 kb and 4.5 kb, and a low abundance transcript of 1.0 kb, among adult tissues of CD-1 mice (FIGURE 13C). The 4.0 kb and 4.5 kb transcripts exhibited different distribution among adult tissues. In the heart, thymus, brain and ovary, these two transcripts exhibited approximately equal distribution. In other tissues, such as the kidney, gut, lung, uterus, and testis, the 4.0 kb transcript was found to be more abundant than the 4.5 kb transcript. Furthermore, *Vezf1* expression in the uterus showed no changes during pregnancy, as shown by the Northern analysis of uteri at day 2.5 or day 16.5 of pregnancy (lanes labeled UTE2 and UTE16 in FIGURE 13C). In E16.5 embryos, no increased *Vezf1* expression was observed in the fetal liver (lane labeled FL in FIGURE 13C), and there was increased expression in the placenta (lane labeled PLAC in FIGURE 13C) which may be

attributed to the vascularity of placental tissue. The fact that the *Vezf1* cDNA characterized above has 3645 nucleotides, and is therefore smaller than the 4.0 kb transcript detected by Northern analysis, may be due to additional 5' UTR sequences in the endogenous transcript.

Conservation of *Vezf1* among species. "Zoo blot" analysis was used to determine whether *Vezf1*-related genes were present in various vertebrate and invertebrate species. Genomic DNA from yeast, *C. elegans*, aplysia, earthworm, *Drosophila*, sea urchin, zebrafish, *Xenopus laevis*, chicken (*Gallus*), mouse and human was digested with BamHI, EcoRI, or HindIII, respectively, and was resolved by agarose gel electrophoresis and transferred to nitrocellulose membrane. The resulting blots were hybridized under low stringency conditions to a *Vezf1* cDNA probe corresponding to nucleotides 697-1444 (probe B in FIGURE 8). Strongly hybridizing fragments were found in chicken, mouse, and human genomic DNA (FIGURE 14B). No significant signals were detected in other DNAs (FIGURE 14A and 14B). Two highly related genes appeared to be present in chicken, mouse and human genomes, and, in human DNA, several additional sequences with reduced homology were present. These results suggest that *Vezf1* is a member of a small family of genes which are conserved in higher vertebrates. The *Vezf1*-related sequences were only detected in those species with yolk sac membrane, which is consistent with the observed first appearance of *Vezf1* gene expression during embryogenesis. Thus, *Vezf1* may serve an important function during yolk sac formation.

Chromosomal mapping of the *Vezf1* locus. A DNA panel obtained from the Jackson Laboratory community resource (C57BL/6J x *M. spretus*)F1 x *M. spretus*) interspecific backcross (Rowe et al., 1994, Mamm. Genome 5:253-274) was used to map the *Vezf1* locus using PCR techniques. A haplotype figure from the Jackson BSS backcross ((C57BL/6JEi x

SPRET/Ei) x SPRET/Ei) indicated that the proximal region of chromosome 2 contains loci linked to *Vezf1* (FIGURE 15A and 15B). *Vezf1* was found to cosegregate with Lhx3, a lim-containing homeoprotein, and genes encoding mouse Notch 1, retinoid receptor alpha subunit (Rxrα), and brain alpha spectrin (Spnα2). *Vezf1* maps adjacent to Abelson proto-oncogene (Abl), adenylate kinase-1(Ak1), endoglin (Eng), erythrocyte band 7.2 (Epb7.2), 78 kd glucose regulated protein (grp78), gelsolin (Gsn), complement component C5 (Hc), and a proto-oncogene homeobox gene (Pbx3). This region of the mouse genome has extensive homology to human chromosome 9q. Indeed, most of the above-mentioned genes were mapped to human chromosome 9q, and some of them have been associated with inherited diseases and disorders. For example, the endoglin gene is associated with hereditary haemorrhagic telangiectasia type 1, an autosomal dominant vascular dysplasia (McAllister et al., 1994, Nature Genet. 8:345-351). However, a human EST clone corresponding to the human DB1 gene has recently been mapped to chromosome 22g. This region is known to map several human vascular and hematopoietic disorders.

Various publication are cited herein, the contents of which are hereby incorporated by reference in their entireties.